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# Non-linear pharmacodynamics in a non-viral gene delivery system: Positive non-linear relationship between dose and transfection efficiency

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**Abstract**

**A remarkable non-linearity was found between dose and transfection activities of non-viral gene delivery systems, such as a Lipofectamine/DNA complex and an octaarginine-modified multifunctional envelope-type nano device (R8-MEND). We measured the nuclear delivery of pDNA to distinguish the non-linearity in intracellular pharmacokinetics or pharmacodynamics after transfection with R8-MEND at different doses. A remarkable positive non-linearity was found in the pharmacodynamics when the dose was increased. Even dummy pDNA enhanced the efficiency of transcription and/or translation per pDNA in the nucleus, but empty liposomes did not. These results suggest the importance of controlled pharmacodynamics as well as the importance of intracellular pharmacokinetics for the rational design of non-viral gene delivery systems.**

*Keywords:* non-viral gene delivery system, intracellular pharmacokinetics, pharmacodinamics, synergism

## 1. Introduction

It is important to clarify the rate limiting step for the intracellular pharmacokinetics (PK), in terms of the rational development of efficient non-viral gene delivery systems [1]. We recently, developed a quantitative method for evaluating each intracellular PK step, including internalization, endosomal escape, the nuclear delivery of a non-viral gene delivery system, using confocal laser scanning microscopy [2]. The findings showed that Lipofectamine PLUS (LFN) was capable of efficiently delivering plasmid DNA (pDNA) to cells via endocytosis, escape from endosomes, reaching the nucleus within 1 hr after transfection, which corresponds to the rate reported for Adenovirus [2]. Based on this methodology, we compared the intracellular PK of pDNA between LFN and Adenovirus. An 8,000-fold difference was found in terms of the number of DNA between LFN and Adenovirus that are required to produce the same transfection activities. Surprisingly, this 8,000-fold difference principally resulted, not from intracellular PK, but from pharmacodynamics (PD) [3].

On the other hand, the importance of PD has already been discussed by Tachibana et al. [4]. They measured the nuclear delivery of pDNA and transfection activities after transfection with different doses of cationic lipids. A remarkable saturation was found between the amount of nuclear pDNA delivered and transfection activities, suggesting that the PD could be a rate limiting step for efficient gene delivery. Under these circumstances, in the present study, a remarkably positive non-linear relationship between dose and gene expression was found for non-viral gene delivery systems. We quantified the nuclear delivery of pDNA by nuclear isolation, followed by real-time PCR to distinguish the non-linearity in the intracellular PK or PD. We introduced a novel method for isolating pDNA in the nuclear fraction without contamination by positively charged complexes of pDNA. As a result, a positive non-linear relationship was observed in the PD of pDNA, but not in the intracellular PK. This positive

non-linearity appears to result from interactions between pDNA in the nucleus, since even dummy pDNA is capable of enhancing the transfection activities without altering the nuclear delivery.

## 2. Materials and Methods

### *2.1. Materials*

Dioleoyl phosphatidylethanolamine (DOPE) was purchased from AVANTI Polar Lipids Inc (Alabaster, AL). Poly-L-lysine (PLL, M.W. 27,400) and cholesteryl hemisuccinate (CHEMS) was obtained from SIGMA-Aldrich Co. (St. Louis, MO). Lipofectamine and PLUS reagent were purchased form Invitrogen Co., (Carlsbad, CA). Stearyl octaarginine (STR-R8) was synthesized as described previously [5]. The pDNAs pCMV-luc (7037 bp) encoding luciferase and pCMV-EGFP (6199 bp) encoding the enhanced green fluorescent protein (EGFP) were prepared by EndFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). NIH3T3 cells were obtained from the American Type Culture Collection (Manassas, VA).

### *2.2. Preparation of R8-modified multifunctional envelope-type nano device (R8-MEND)*

The multifunctional envelope-type nano device (MEND) consists of condensed DNA core and lipid envelope modified with functional devices such as STR-R8 [1]. The MEND was prepared by the lipid film hydration method described in a previous report [6]. In a typical experiment, DNA dissolved, in 10mM HEPES buffer (pH 7.4) (0.1 mg/ml), was mixed with a solution of PLL (0.1 mg/ml) to condense the pDNA under vortexing at a nitrogen/phosphate (N/P) ratio of 2.4. A 0.25 ml of aliquot of the condensed pDNA suspension was then added to the lipid film containing 137.5 nmol lipids (DOPE/CHEMS = 9:2 (molar ratio)), followed by incubation for 10 min to hydrate the lipids. The final concentration of lipid was 0.55 mM. The hydrated mixture was sonicated to produce the MEND by coating the condensed pDNA with lipids for 1 min in a bath-type sonicator (85 W, Aiwa Co., Tokyo, Japan). A STR-R8 solution (5 mol % of lipids) was added to the suspension to attach the R8 peptide to the envelope of the MEND (R8-MEND), and the mixture was incubated for 30 min at room temperature.

Empty R8-modified liposomes were prepared by the same procedure except for the condensation and packaging of pDNA.

*2.3. Transfection assay*

A 4 μl aliquot of PLUS reagent was mixed with 121 μl of DMEM containing 0.4μg of pDNA, without serum and antibiotics (DMEM(-)), and incubated for 15 min at room temperature. A 1 μl aliquot of Lipofectamine was suspended with 124 μl of DMEM(-). The Lipofectamine suspension was then added to the PLUS/pDNA mixture, and further incubated for 15 min at room temperature. Samples containing 0.04 μg or 0.4 μg of pDNA suspended in 0.25 ml of DMEM(-) were added to 4 x $10^4$ cells (1 pg pDNA/cell or 10 pg pDNA/cell), and incubated for 3 h at 37 $^oC$. Next, 1 ml of DMEM containing 10 % fetal calf serum was added to the cells, followed by a further 45 h incubation. The cells were then washed, and solubilized with reporter lysis buffer (Promega, Madison, WI). The luciferase reaction was initiated by the addition of 100 μl of lusiferase assay reagent (Promega, Madison, WI) to 20 μl of the cell lysate, and measured by means of a luminometer (Luminescencer-PSN, ATTO, Japan). The amount of protein in the cell lysate was determined using a BCA protein assay kit (PIERCE, Rockford, IL). Data are reported as the mean ± standard deviation (n=3).

*2.4. Quantification of pDNA in nucleus*

Samples containing 0.2 μg or 2 μg of pDNA suspended in 1.25 ml of DMEM(-) was added to 2 x $10^5$ NIH3T3 cells, and incubated for 3 h at 37 $^oC$. Next, 2 ml of DMEM containing 10 % fetal calf serum was added to the cells followed by a further incubation for 3 h. The cells were then washed twice with PBS, and collected by trypsinization. The collected cells were suspended with 100 μl of CellScrub Buffer (Gene Therapy Systems Inc.) to remove the cell surface-bound lipoplex or MEND, and

gently shaken at 4 $^{o}$C for 30 min. The cell suspension was then centrifuged at 2,000 rpm for 3 min at 4 $^{o}$C, and the precipitate was subjected to a real time PCR method as the whole cell sample. On the other hand, for nuclear extraction, the trypsinized cells were centrifuged, and the precipitated cells were suspended in 187.5 μl of CellScrub Buffer and 62.5 μl of cell lysis solution (2% IGEPAL CA630, 40 mM NaCl, 12 mM $MgCl_2$ and 40 mM Tris-HCl, pH 7.4) was then further applied. The suspension was centrifuged at 9,200 x *g* for 2 min at 4 $^{o}$C, and the supernatant was removed. This handling was repeated 3 times. The precipitate was then washed with PBS, and subjected to a real time PCR method as a representative nucleus sample. For the real time PCR method, pDNA in whole cell or nucleus samples was extracted using GenElute Mammalian Genomic DNA Miniprep Kit (SIGMA-Aldrich Co.) following the recommended protocol. The real time PCR was performed using a PCR apparatus (7500 Real Time PCR System, Applied Biosystems Co.) under the following conditions; the reaction mixture consisted of 5 μl of diluted sample DNA solution, 5 pmol of two types of primers (luc(+): GGTCCTATGATTATGTCCGGTTATG and luc(-): ATGTAGCCATCCATCCTTGTCAAT, or Beta-actin-FW694: AGAGGGAAATCGTGC GTGAC and Beta-actin-RV811: CAATAGTGATGACCTGGCCGT) and 12.5 μl of SYBR Green Realtime PCR Master Mix (TOYOBO co.). Denaturation at 95 $^{o}$C for 15 sec, and annealing/extension at 60 $^{o}$C for 1 min was then performed. The denaturation/anneling cycle was repeated 40 times.

## 3. Results and Discussion

In the present study, we focused on the non-linearity in the dose and transfection activities for non-viral gene delivery systems. As can be seen in previous reports, a non-linear relationship between the dose of pDNA and the extent of gene expression has been reported for various non-viral gene delivery systems, such as a Lipofectamine [7, 8], DC-Chol/DOPE [9], DMRIE/DOPE [10], TFL-3/Chol [4] and LMD [11]. However, the origin of this non-linearity *i.e.*, intracellular PK or PD, is not known with certainty. In an initial experiment, we examined the relationship between the dose of pDNA and transfection activities in case of LFN and luciferase-encoding pDNA in NIH3T3 cells. As shown in Fig. 1 (A), the luciferase activity increased with increasing dose but this increase was exponential and not linear. A remarkable positive non-linear relationship was found between the dose and gene expression, i.e., when the dose of DNA was 5 pg/cell, the luciferase activity ($2.5x10^9$) was 60 times higher than that of 1 pg/cell ($4.2x10^7$). However, the double amount of DNA (10 pg/cell) induced 360 times higher activity ($1.5x10^{10}$) than 1 pg/cell. This non-linear increase of luciferase activity at higher value of pDNA was not expected and could also not be explained. A similar result was also observed for the R8-MEND, a recently developed non-viral gene delivery system [6] (Fig. 1 (B)). There are many steps involved in the intracellular trafficking of pDNA after endocytosis to reach nucleus as shown in Fig. 2. Saturation can usually be seen in internalization by endocytosis, lysosomal degradation, and nuclear translocation through a nuclear pore complex. Therefore, lysosomal degradation could be a factor in the positive non-linearity shown in Fig. 1, since an increased dose can saturate lysosomal degradation thus leading to enhanced transfection activities. Saturation in endocytosis or nuclear translocation cannot be a cause of the positive non-linearity, since saturation in these processes would lead to a decreased cellular uptake/nuclear delivery.

Based on above considerations, we examined the effect of a dummy R8-MEND, which encapsulated pDNA encoding EGFP but not luciferase, on the luciferase activity of R8-MEND encapsulating luciferase pDNA. In addition, we also examined the effect of co-existence of empty R8-modified liposomes, which consist of the same lipids and STR-R8 without condensed pDNA, on the transfection activity of the R8-MEND. As shown in Fig. 3, the transfection activity of the R8-MEND at 10 pg pDNA/cell (High dose) was 230 times higher than that at 1 pg DNA/cell (Control), which represents a 23-fold nonlinear increase. Surprisingly, the transfection activity of the R8-MEND at 1 pg pDNA/cell with 9 pg dummy pDNA (+Dummy) showed 40 times enhanced transfection activities compared to the control. Similarly, empty liposomes enhanced the transfection activity by 6 fold, although the increment was smaller than that of the dummy pDNA. Similar results were also observed for other non-viral gene delivery systems, such as LFN and DOTAP/DOPE lipoplex, i.e., dummy LFN and dummy DOTAP/DOPE showed 3.5 times and 117 times enhanced transfection activities, respectively. These results suggest that the high dose, dummy pDNA and empty liposomes are able to saturate lysosomal degradation, which leads to the enhanced transfection activities, because degradation abilities of DNases and lipase could be saturated by the excess amounts of pDNA and lipids, respectively.

To understand the non-linearity more clearly, we divided the overall process into intracellular PK (endocytosis, endosomal escape and nuclear delivery) and PD (nuclear stability, transcriptional efficiency, translational efficiency, etc.). The efficiency of intracellular PK can be estimated by measuring the cellular uptake and nuclear delivery of pDNA. Previously, Pollard et al. reported the relationship between copy number of pDNA in nucleus and gene expression [12]. They however did not pay attention to the efficiency of PD in nucleus. The efficiency of PD can be estimated by the transfection activities divided by the nuclear delivered pDNA. Therefore, we measured the nuclear

pDNA after transfection. However, difficulties were encountered in determining the pDNA in the nuclear fraction without contamination of the pDNA in other fractions such as the cytosolic and/or endosomal/lysosomal fraction. When nuclear fractionation was performed without treatment with CellScrub buffer, the percent of nuclear association of pDNA to the total cellular uptake at 1 h was estimated to be more than >70%, when transfected with LFN. This is inconsistent with our recent results showing that the average nuclear association was approximately 20%, when estimated by confocal image-assisted 3-dimensionally integrated quantification (CIDIQ) [2]. Therefore, nuclear delivered pDNA after subcellular fractionation was greatly overestimated presumably due to contamination of the nuclear fraction by cytoplasmic lipoplex via electrostatic interactions during the nuclear isolation process. To avoid this contamination, the CellScrub buffer was used together with the cell lysis buffer, to absorb the cytoplasmic lipoplex. As a result, the percent of nuclear association was decreased to a value (17%) that was comparable to that estimated by CIDIQ, indicating that the CellScrub buffer is useful in avoiding non-specific contamination of the nuclear fraction by cytoplasmic polyplex.

As summarized in Table 1, a slight (within one order of magnitude) difference was detected in the nuclear pDNA between control and other conditions, which suggests similar intracellular PK. These results show that the nuclear delivery of pDNA cannot be the cause of the positive non-linearity, which excludes the intracellular PK as the candidate for the non-linearity.

The transfection activities divided by the nuclear delivered pDNA represents the efficiency of the PD. Fig. 4 shows data on the effect of the high dose, dummy pDNA and empty liposomes on the intracellular PK (open column) and PD (closed column). Remarkable non-linear effects in PD by the high dose and the dummy pDNA were observed, where the synergistic effects were more than one order of magnitude

compared to the control. On the other hand, slight increases in intracellular PK were observed by high dose, dummy pDNA and empty liposomes. These results clearly indicate that the origin of the positive non-linearity was primarily inside the nucleus.

The enhanced efficiency in PD for the high dose and dummy pDNA suggests that the total amount of pDNA in the nucleus plays an important role in determining the efficiency of PD. The condensed pDNA released from the MEND could be decondensed by the substitution with negatively charged components such as genomic DNA or RNA. As a result, pDNA could be released in the nucleus. The released pDNA however could be bound and inactivated by histone as heterochromatine. In the presence of excess pDNA, the amount of transcriptable released form of pDNA could increase due to saturable binding between excess pDNA and histones in the nucleus. Methylation of the pDNA can also be an inactivation mechanism and a possible cause of the positive non-linearity observed in the PD [13, 14]. Since the saturation of this process by an increased amount of pDNA in the nucleus can enhance transfection activities, methylation could be the cause of the positive non-linearity. Further study will be required to identify the factors responsible for the positive non-linearity.

In the present study, we found a remarkable positive non-linear relationship between dose and transfection activities in non-viral gene delivery systems such as LFN and R8-MEND. Surprisingly, a synergetic relationship between the nuclear delivered pDNA and transfection efficiency in the nucleus was found. A slight non-linearity in the intracellular PK was found. These results clearly demonstrate the importance of recognizing non-linear PD in the rational development of non-viral gene delivery systems.

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**References**

[1] H. Kamiya, H. Akita, H. Harashima, Pharmacokinetic and pharmacodynamic considerations in gene therapy. Drug Discov. Today 21 (2003) 990-996.

[2] H. Akita, R. Ito, I.A. Khalil, S. Futaki, H. Harashima, Quantitative three-dimensional analysis of the intracellular trafficking of plasmid DNA transfected by a nonviral gene delivery system using confocal laser scanning microscopy. Mol. Ther. 9 (2004) 443-451.

[3] S. Hama, H. Akita, R. Ito, H. Mizuguchi, T. Hayakawa, H. Harashima, Quantitative comparison of intracellular trafficking and nuclear transcription between viral and non-viral vectors. submitted for publication.

[4] R. Tachibana, H. Harashima, N. Ide, S. Ukitsu, Y. Ohta, N. Suzuki, H. Kikuchi, Y. Shinohara, H. Kiwada, Quantitative analysis of correlation between number of nuclear plasmids and gene expression activity after transfection with cationic liposomes. Pharm. Res. 19 (2002) 377-381.

[5] S. Futaki, W. Ohashi, T. Suzuki, M. Niwa, S. Tanaka, K. Ueda, H. Harashima, Y. Sugiura, Stearylated arginine-rich peptides: a new class of transfection systems. Bioconjug. Chem. 12 (2001) 1005-1011.

[6] K. Kogure, R. Moriguchi, K. Sasaki, M. Ueno, S. Futaki, H. Harashima, Development of a non-viral multifunctional envelope-type nano device by a lipid film hydration method. J. Control. Release 98 (2004) 317-323.

[7] P.J. Shih, K. Evans, K. Schifferli, V. Ciccarone, F. Lichaa, M. Masoud, J. Lan, P. Hawley-Nelson, High efficiency transfection with minimal optimization using the lipofectAMINE PLUS reagent. Focus 19 (1997) 52-56.

[8] V. Ciccarone, V. Chu, K. Shifferli, J.P. Pichet, P. Hawley-Nelson, K. Evans, L. Roy, S. Bennett, LipofectAMINE 2000 reagent for rapid, efficient transfection of eukaryotic cells. Focus 21 (1999) 54-55.

[9] N.J. Caplen, E. Finrade, F. Sorgi, X. Gao, D. Gruenert, D. Geddes, C. Coutelle, L. Huang, E.W.F.W. Alton, R. Williamson, In vitro liposome-mediated DNA transfection of epithelial cell lines using the cationic liposome DC-Chol/DOPE. Gene Ther. 2 (1995) 603-613.

[10] J. Zabner, A.J. Fasbender, T. Moninger, K.A. Pollinger, M.J. Welsh, Cellular and molecular barriers to gene transfer by a cationic lipid. J. Biol. Chem. 270 (1995) 18997-19007.

[11] T. Tagawa, M. Manvell, N. Brown, M. Keller, E. Perouzel, K.D. Murray, R.P. Harbottle, M. Tecle, F. Booy, M.C. Brahimi-Horn, C. Coutelle, N.R. Lemoine, E.W.F.W. Alton, A.D. Miller, Characterisation of LMD virus-like nanoparticles self-assembled from cationic liposomes, adenovirus core peptide μ (mu) plasmid DNA. Gene Ther. 9 (2002) 564-576.

[12] H. Pollard, J.-S. Remy, G. Loussouarn, S. Demolombe, J.-P. Behr, D. Escande, Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J. Biol. Chem. 273 (1998) 7507-7511.

[13] M. Curradi, A. Izzo, G. Badaracco, N. Landsberger, Molecular mechanisms of gene silencing mediated by DNA methylation. Mol Cell Biol 22 (2002) 3157-3173.

[14] R.A. Irvine, I.G. Lin, C.L. Hsieh, DNA methylation has a local effect on transcription and histone acetylation. Mol. Cell Biol. 22 (2002) 6689-6696.

**Figure legends**

Figure 1. Positive non-linear relationship between dose and gene expression in (A) LFN and (B) R8-MEND

The effect of the dose of pDNA on the transfection activities on the LFN (A) or R8-MEND (B) was examined. The dose of pDNA was altered from 1 pg/cell to 10 pg/cell in NIH3T3 cells. The X-axis means the applied amount of luficerase pDNA per cell. Luciferase activities were measured 48 h after transfection.

Figure 2. Schematic image of intracellular PK and PD of pDNA with a non-viral gene delivery system

Packaged (or complexed) pDNA is internalized via endocytosis, leading lysosomal degradation. Once escaping from endosome/lysosome either in condensed form or free form, the pDNA must then pass through the nuclear membrane. The condensed pDNA in the nucleus must then be decondensed to free pDNA before transcription. The transcripted messenger RNA is effluxed to cytosol for translation.

Figure 3. The effect of high dose, dummy pDNA and empty liposomes on the transfection activities by the R8-MEND

The luciferase pDNA in R8-MEND was fixed at 1 pg/cell except for the High dose (10 pg/cell). In the case of the Dummy, 9 pg of EGFP pDNA was encapsulated in the R8-MEND. In the case of Empty liposomes, empty R8-liposomes (124 nmol) were co-administrated with the R8-MEND. Luciferase activities were measured at 48 h after transfection. Data were represented as mean ± S.D. (n=3).

Figure 4. Distinction in non-linearity between intracellular PK and PD

The intracellular PK (open column) is shown as the ratios of the average of intranuclear

pDNA per the average of whole cell pDNA, and the PD (closed column) was estimated by dividing the average of transfection activities (Fig. 3) by the average number of pDNA copies in the nucleus (Table 1).

Table 1. Numbers of pDNA copies in whole cells and nucleus after transfection using the R8-MEND.

| | Whole cell (copies/cell) | Nucleus (copies/cell) |
|---|---|---|
| Control | $5.8\times10^3 \pm 0.5\times10^3$ | $1.6\times10^3 \pm 0.3\times10^3$ |
| High dose | $8.7\times10^4 \pm 1.8\times10^4$ | $3.1\times10^4 \pm 1.1\times10^3$ |
| Control + dummy R8-MEND | $8.0\times10^3 \pm 1.1\times10^3$ | $4.7\times10^3 \pm 1.4\times10^3$ |
| Control + empty R8-liposome | $1.5\times10^4 \pm 1.2\times10^3$ | $5.7\times10^3 \pm 1.5\times10^3$ |

Data are shown as mean±S.D. (n=3).

Figure 1.

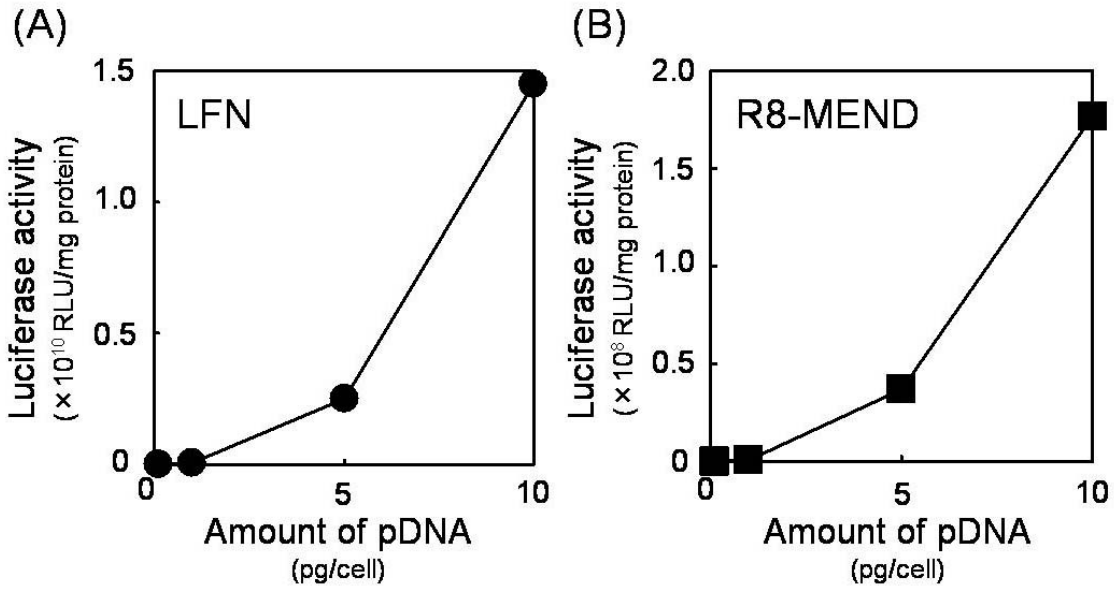


Figure 2.

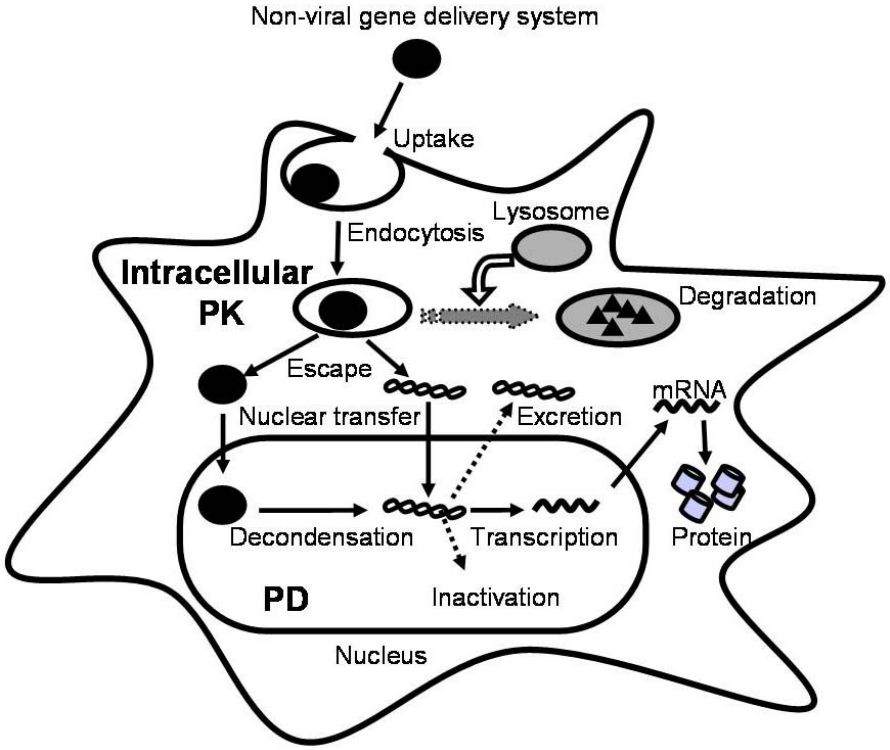

Figure 3.

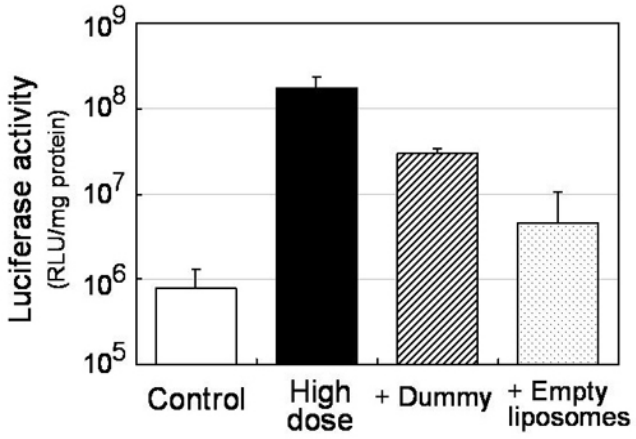


Figure 4.